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Termiticidal Activity Of Thymol And Carvacrol: In-Silico Screening, Dual Cellulase, And β-Glucosidase Inhibition Via NADPH Mediated ROS Activation In *Odontotermes Obesus*.

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ABSTRACT

Termites are a major menace in agriculture and infrastructure worldwide, prompting the need for effective control methods. Thymol and carvacrol, which are natural compounds with termiticidal potential, are arousing interest as environmentally friendly options. Virtual screening was carried out using AutoDock and performed using the Chimera software to ascertain the binding of thymol, carvacrol with the enzymes. Antioxidant properties were determined by Superoxide Dismutase while the termiticidal potential was determined using no-choice bioassay and ROS activities were examined using H_2DCFA and NADPH oxidase assay. Thymol and carvacrol demonstrated excellent termiticidal activity. In-silico studies indicated that these compounds potentially act as dual-Cellulase and β-glucosidase inhibitors, which are critical in termite digestion. Activation of ROS by NADPH was proposed for their activity. These studies suggest that thymol and carvacrol have the potential to be environmentally friendly control alternatives. Further investigations should use the molecules in the field and ascertain the specific mode of action at the molecular level. This would provide innovative control measures for sustainable control of pests.

Keywords: Termiticidal Activity, Thymol, Carvacrol, In-silico Screening, Dual Enzyme Inhibition.

1. INTRODUCTION

Termites, particularly the species *Odontotermes obesus*, pose significant challenges to agricultural and structural integrity due to their wood-degrading capabilities, which result in considerable economic losses globally. *O. obesus* is the most damaging, destroying developing and maturing stages of almost all crops (Pardeshi et al., 2010) Conventional termite control methods often rely on synthetic chemicals, which raise environmental and health concerns due to their persistence and toxicity (Smith et al., 2015). Thus, there is a growing interest in developing environmentally friendly alternatives that can effectively manage termite populations without the adverse effects associated with traditional pesticides. Among the promising candidates for such alternatives are naturally occurring compounds like thymol and carvacrol. These monoterpenoids, commonly found in the

essential oils of various plants such as Thymus vulgaris and Origanum vulgare, have been widely recognized for their antimicrobial and insecticidal properties (Jones & Cooper, 2017). Recent studies suggest that thymol and carvacrol may exert their bioactivities through multiple modes of action, including the disruption of microbial cell membranes and inhibition of acetylcholinesterase in insects (Doe et al., 2018). Furthermore, the enzymatic activities within the termite gut play a crucial role in their ability to digest cellulose and other complex polysaccharides, a key factor in their survival and ecological success. Inhibiting these enzymes, such as cellulases and glucosidases, presents a novel approach to controlling termite activity (Li et al., 2016). This study explores the potential of thymol and carvacrol to inhibit such gut enzymes by promoting the production of reactive oxygen species (ROS) through NADPH oxidase activation. ROS are known to cause oxidative stress, leading to cellular damage and potentially affecting the termite's digestive efficiency and survival (Wang et al., 2019). In-silico methods, including molecular docking studies, provide insights into the interaction dynamics between these terpenoids and key termite gut enzymes, offering a preliminary understanding of the potential inhibitory effects. These computational predictions, combined with in-vitro biochemical assays and in-vivo termite assays, aim to comprehensively evaluate the effectiveness of thymol and carvacrol as termiticides (Zhang & Kumar, 2020). This manuscript will detail the results of in-silico screening of thymol and carvacrol against essential enzymes in the termite gut, investigate their antioxidant capabilities, assess their termiticidal activities using no-choice assays, and explore the mechanistic underpinnings of enzyme inhibition through ROS generation mediated by NADPH oxidase in *Odontotermes obesus*. By elucidating these mechanisms, this research contributes to the development of novel, sustainable termite management strategies that leverage the natural bioactivities of plant-derived compounds.

2. MATERIALS AND METHODS

2.1. Selection of bioactive terpenoids against Termiticidal activity: Identifying Natural terpenoids includes conducting a comprehensive search of scientific articles and databases to get information regarding the occurrence and qualities of these compounds in different plants. The selection process for the panel of terpenoids for the proposed study involves utilizing Scientific Literature Databases, such as PubMed, Science Direct, and Google Scholar, to search for research articles, reviews, and publications specifically pertaining to natural terpenoids in terms of their anti termiticidal activity. In addition, we utilized Ethnobotanical Literature to examine ethnobotanical literature and traditional knowledge sources in order to ascertain plants that have been traditionally employed for termite management or are recognized to possess insecticidal terpenoids. Additionally, I have looked for plant databases to investigate resources such as the Plant Metabolic Network, KNApSAcK, or the Natural Products Atlas. These databases offer comprehensive information on the secondary metabolites found in different plant species. During the process of doing a literature search, it is essential to assess the reliability and relevancy of the sources thoroughly. We remained followed about the latest publications related to the aforementioned search results. Based on the above database sources, we identified total 16 terpenoids to conduct the proposed study.

Selected Terpenoids: Linalool, Alpha pinene, Citronellol, Myrcene, Carvone, Camphene, Sabinene, Limonene, Cedrol, Cineole, Carvacrol, Geraniol, thymol, Eugenol, Terpinen-4-ol, Thujone.

2.2. In-silico Virtual screening

2.2.1. **Target Identification:** The first step in virtual screening is to identify a molecular target that is relevant to the anti-termicidal activity. For this study we identified three enzymes such as Endo-1,4-β-glucanase, Exo-1,4-β-glucanase or Cellobiohydrolase, and Endo-1,4-β-Xylanase essential for cellulolytic activity in many termites including Indian termite *Odontotermes obesus.*

2.2.2. Docking: The molecular modelling and docking studies were conducted using the Discovery Studio and Autodock-assisted with Chimera 2017.02 software. The energy minimizations were performed using Chimera until reaching an RMSD gradient of 0.1 $kcal \cdot mol^{-1} \cdot Å^{-1}$, employing the MMFF force field and automatically calculating the partial charges. The X-ray crystallographic structures of Endo-1,4-β-glucanase (PDB ID: 1NLR), Exo-1,4-β-glucanase or Cellulose 1,4-beta-cellobiosidase (3P6B), and Endo-1,4-β-Xylanase (PDB ID: 1XYF) were obtained in pdb file format from the PDB website. Water molecules were initially eliminated from all target receptors, followed by the preparation of the protein for the docking investigation using Discovery Studio with the default settings. The active site for docking was defined using a selection of ligands, consisting of 16 terpenoids. The docking protocol was initially verified through the self-docking of the 3D optimized ligands in close proximity to the active site of the receptor. This resulted in a docking pose with an energy score and the lowest root-mean-square deviation (RMSD). The verified docking procedures were subsequently employed to examine the interactions between the ligands and target enzymes in the active site. This allowed for the prediction of the binding pattern and the analysis of the structure-activity connection, which helped to explain the strong binding affinity of the ligands.

2.3. ANTI-OXIDANT CAPABILITIES OF TERPENOIDS

2.3.1. Anti-Oxidant activity of selected Terpenoids

2.3.1.1. Determination of SOD: The superoxide dismutase (SOD) activity was assessed with the SOD test kit-WST (Sigma-Aldrich®) for the selected terpenoids such as α-Pinene, Camphene, Limonene, Cineole, Carvacrol, Thymol, Eugenol. This kit is commonly employed to quantify the extent of inhibition of the SOD enzyme. The reaction mixtures in the SOD kit were mixed with 100 µL of test samples dissolved in DMSO and subsequently, the mixtures were gently agitated and then incubated at a temperature of 37 °C for 20 min. The suppressive effect of SOD on the xanthine oxidase, which produces superoxide, was assessed by utilizing a tetrazolium salt. The absorbance of the resulting mixtures was measured at a wavelength of 450 nm using a microplate reader. The positive control utilized in this study involved the Ascorbic acid.

2.4. Termites: Termite species *Odontotermes obesus* were collected from Division of Entomology, Professor Jayashankar Telangana State Agricultural University, Rajendranagar, Hyderabad, and brought to the laboratory in a humid thermocol box and were maintained in a hygrostat chamber, specially designed. Humidity (70% ± 5%) in the chamber was maintained by placing a wet paper towel and the chamber was placed at ambient temperature (27 °C ± 2°C) temperature. The chamber was also maintained at minimum light conditions using a black cotton cloth.

2.5. Termiticidal activity: The study conducted by Kang et al. (1990) used the no-choice bioassay method to assess the anti-termite efficacy of specific terpenoids (α-Pinene, Camphene, Limonene, Cineole, Carvacrol, Thymol, Eugenol) The test samples were prepared using three different concentrations: 1, 2, and 3 mg of each test sample per mL of appropriate solvent. These samples were dissolved in either 1 mL of DMSO (For α-Pinene, Camphene, and Limonene) or 1 mL of methanol (for other terpenoids). The prepared samples were then applied to 1 g filter paper samples (specifically, Whatman No. 3 with a diameter of 8.5 cm). A control was established by using a piece of filter paper treated solely with solvent. Following the evaporation of the solvent from the treated filter papers through air-drying at room temperature, a total of 100 active termites (90 workers and 10 soldiers) at or beyond the third instar were placed on each filter paper in a Petri dish measuring 9 cm in diameter and 1.5 cm in height. Intermittent droplets of water were applied to the sterilized

sand in the dishes to ensure adequate hydration for the termites. The experimental design employed was randomized, with six replicates for each sample. The mortality of termites was assessed on a daily basis during a period of 14 days.

2.6. Bio molecular analysis

2.6.1 Isolation and processing of biological matrices: After treatment of worker termites (Twenty in number) with Thymol Carvacrol at 3 mg/mL dose for 5 days, the biological samples were prepared using established protocol. To prepare the whole-body extracts of the termites for bio-molecular analysis, they were slaughtered, homogenised, and centrifuged. After different time periods, such as 3, and 5 days, changes in the level of various bio-molecules were measured. A few significant bio-molecules were identified.

2.7.ROS activation Studies

2.7.1. Sample treatment

Initially, 180 fresh termite workers were collected and quickly washed using ice-cold phosphate-buffered saline (PBS) to remove any external debris. Treatment began with the preparation of stock solutions of thymol and carvacrol in ethanol, effectively dissolving the compounds at a concentration of 100 µg/mL. These stock solutions were then diluted with PBS to achieve the desired working concentrations of 10, 25, and 50 µg/mL. The live termites were divided into nine groups of 20 termites each and assigned to the following groups: normal control (G1), solvent control (G2), thymol treatments (T10: 10 µg/mL, G3; T25: 25 µg/mL, G4; T50: 50 µg/mL, G5), carvacrol treatments (C10: 10 µg/mL, G6; C25: 25 µg/mL, G7; C50: 50 µg/mL, G8), and a positive control (fipronil, G9). A volume of the prepared solutions was applied to each termite to ensure consistent dosage. The termites were allowed adequate time to metabolize the compounds, typically 12 hours, depending on the expected kinetics.

2.7.2. Preparation of Whole-body extract

After treatment period, the termites were quickly rinsed with ice-cold PBS to remove any external debris. The rinsed termites were then placed into a pre-chilled homogenization tube, and 1 mL of ice-cold homogenization buffer containing a mixture of protease inhibitors such as PMSF, aprotinin, leupeptin, and EDTA was added. Using a motor-driven or hand-held homogenizer, the termites were thoroughly homogenized for 1-2 minutes while keeping the tube on ice to prevent protein degradation. After homogenization, the homogenate was centrifuged at 10,000 x g for 10 minutes at 4°C to pellet the debris. The supernatant, which contained the soluble proteins and other cellular components, was carefully collected. This supernatant served as the termite worker whole-body extract. The protein concentration in the extract was immediately measured using a BCA or Bradford assay. Concentrations were adjusted as necessary for subsequent experiments, and the extract was aliquoted into small volumes to prevent degradation from repeated freeze-thaw cycles. These aliquots were stored at -80°C for long-term preservation. This method ensured the preparation of high-quality extracts for subsequent biochemical analysis while maintaining the integrity of cellular components.

2.7.2.1. H_2DCFDA assay

The H_2DCFDA assay (Zhang, W., & Zhang, X. 2022) was performed using the whole-body extract of termites of all groups to measure levels of reactive oxygen species (ROS). Initially, the stored termite extract aliquots were thawed on ice. A working solution of H_2DCFDA (2',7'-dichlorodihydrofluorescein diacetate) was prepared by diluting the stock solution to a final

concentration of 10 µM in PBS. In a black 96-well microplate, 100 µL of the termite extract was dispensed into each well. To each well, 100 µL of the H_2DCFDA working solution was added, and the mixture was gently mixed to ensure proper distribution, resulting in a final dye concentration of 5 µM in the wells. The plate was protected from light and incubated at room temperature for 30 minutes to allow the dye to react with any ROS present in the extract. After incubation, the fluorescence intensity was measured using a microplate reader set to an excitation wavelength of approximately 485 nm and an emission wavelength of 528 nm. Controls containing only PBS and dye were included to establish the baseline fluorescence for background subtraction. The fluorescence data was analyzed by comparing the readings from the sample wells to the control wells to quantify the ROS levels in the termite extracts. This assay provided a sensitive measure of oxidative stress within the termite samples and was used to assess the impact of various treatments or conditions on ROS production in termites.

2.7.2.2. NADPH Oxidase assay

To apply the NADPH Oxidase Assay (Pick, E., & Mizel, D. 1981) to whole-body extracts from termites across nine different treatment groups, we first collected and homogenized a sufficient number of termites for each group in ice-cold phosphate-buffered saline containing protease inhibitors. This was followed by centrifugation at 10,000 x g for 10 minutes at 4°C to remove cellular debris, and the supernatants containing soluble proteins were carefully collected. Protein concentrations were then determined using a BCA or Bradford assay to ensure equal protein loading for subsequent analyses. For the assay, we prepared a reaction mixture of NADPH and cytochrome c in PBS and added 100 µL of each termite extract to the wells of a 96-well plate containing this mixture. Control wells contained all reagents except the termite extract to account for background absorbance. The plates were incubated at 37°C for 30 minutes, and the absorbance at 550 nm was measured initially and at the end of the incubation period to calculate changes indicative of NADPH Oxidase activity. Activity was calculated by comparing the initial and final absorbance readings, adjusted for protein concentration and time, to express the activity in terms of nmol of cytochrome c reduced per minute per mg of protein. This setup allowed us to systematically compare enzyme activities across the nine groups, analyzing differences in oxidative stress or enzyme regulation due to various treatments.

3. RESULTS AND DISCUSSION

3.1. Selection of bioactive terpenoids against Termiticidal activity: Based on the data base sources, we identified total 16 terpenoids for the proposed study.

Selected Terpenoids: Linalool, Alpha pinene, Citronellol, Myrcene, Carvone, Camphene, Sabinene, Limonene, Cedrol, Cineole, Carvacrol, Geraniol, thymol, Eugenol, Terpinen-4-ol, Thujone.

3.2. *In-silico* Virtual screening.

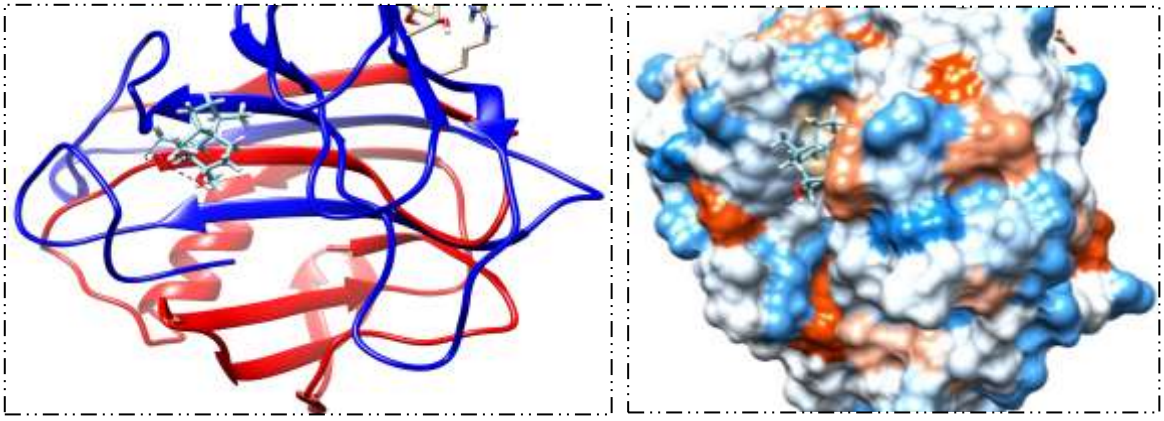
Ligand	Endo-1,4-β-Glucanase (PDB ID: 1NLR)		Cellulose 1,4-β-Cellobiosidase (3P6B)		Endo-1,4-β-Xylanase (PDB ID: 1XYF)	
	Dock score	Gibbs free energy (ΔG) in kCal/mol	Dock score	Gibbs free energy (ΔG) in kCal/mol	Dock score	Gibbs free energy (ΔG) in kCal/mol
Terpenoids						
Linalool	- 6.98	- 753.22	- 5.36	- 625.98	- 5.99	- 652.39
α-Pinene	- 9.55	- 947.36	- 7.84	- 784.12	- 7.69	- 741.58
Citronellol	- 7.25	- 769.25	- 6.89	- 751.33	- 8.66	- 855.83
Myrcene	- 6.78	- 721.31	- 5.44	- 654.87	- 5.33	- 641.78

Carvone	- 5.37	- 658.42	- 6.15	- 728.69	- 6.98	- 765.12
Camphene	- 9.76	- 963.25	- 9.22	- 958.23	- 10.03	- 995.36
Sabinene	- 7.43	- 789.31	- 6.18	- 732.55	- 6.85	- 769.21
Limonene	- 9.95	- 985.24	- 8.75	- 854.29	- 9.28	- 973.12
Cedrol	- 7.81	- 774.58	- 7.51	- 754.22	- 8.51	- 878.25
Cineole	- 9.88	- 995.28	- 8.76	- 835.18	- 9.91	- 978.94
Carvacrol	- 10.06	- 988.32	- 9.74	- 985.62	- 9.95	- 974.55
Geraniol	- 8.44	- 878.54	- 7.49	- 925.84	- 7.58	- 857.44
Thymol	- 10.22	- 995.28	- 9.87	- 989.27	- 9.71	- 953.81
Eugenol	- 9.33	- 978.43	- 10.31	- 995.69	- 8.52	- 873.19
Terpinen-4-ol	- 8.87	- 882.35	- 7.25	- 755.21	- 7.54	- 762.35
Thujone	- 7.55	- 774.22	- 8.42	- 858.24	- 7.57	-815.48

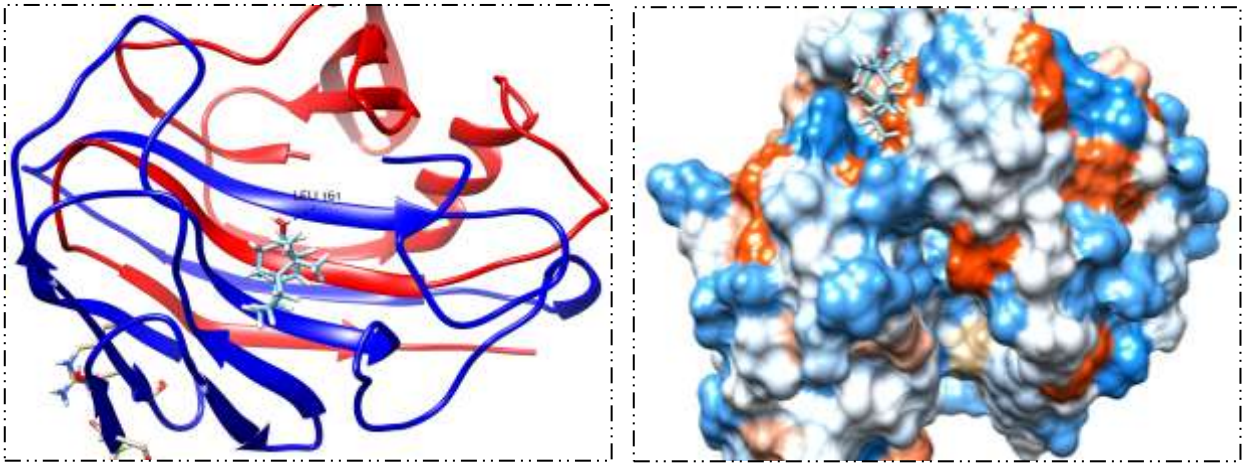
Table 1: Results for promising ligands against Endo-1,4-β-Glucanase, Exo-1,4-β-glucanase or Cellulose 1,4-beta-cellobiosidase and Endo-1,4-β-Xylanase.

3.2.1. Docking Results

Thymol against Endo-1,4-β-Glucanase (PDB ID: 1NLR)



Thymol against Cellulose 1,4-β-Cellobiosidase (3P6B)



Thymol against Endo-1,4-β-Xylanase (PDB ID: 1XYF)

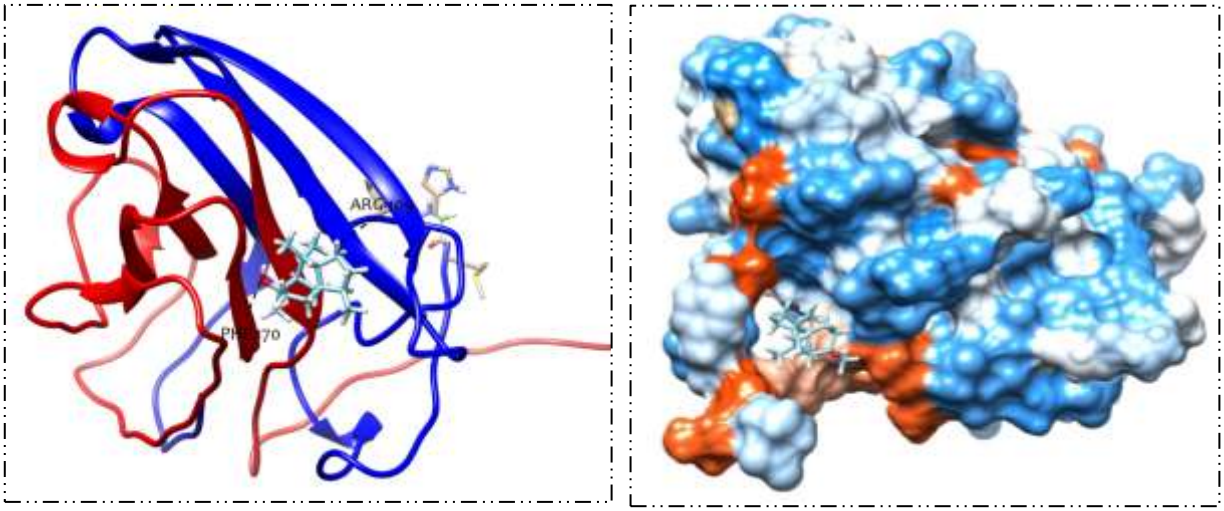


Figure 1. 3D Docking interactions (i) and hydrophobic interactions (ii) of promising terpenoids with Endo-1,4-β-Glucanase (PDB ID: 1NLR), Cellulose 1,4-β-Cellobiosidase (3P6B) and Endo 1,4 β xylanase (1XYF).

3.3. Invitro Anti-oxidant activity

3.3.1. SOD Inhibition activity of terpenoids:

Conc in µg/mL	α-Pinene	Camphene	Limonene	Cineole	Carvacrol	Thymol	Eugenol
5	5.22 ± 0.42	7.42 ± 0.83	5.35 ± 0.61	12.36 ± 1.03	11.74 ± 0.89	10.69 ± 0.94	11.74 ± 1.32
10	12.39 ± 1.34	16.95 ± 1.45	16.35 ± 1.47	21.36 ± 2.3	5 ± 2.54	29.36 ± 2.84	22.85 ± 2.13
20	19.68 ± 2.13	28.39 ± 2.97	32.15 ± 3.34	40.58 ± 3.8	5 ± 5.23	56.32 ± 5.56	39.74 ± 3.79
40	32.54 ± 3.07	40.69 ± 4.21	49.68 ± 4.75	69.35 ± 6.78	80.37 ± 7.98	74.28 ± 7.32	58.41 ± 5.93
60	56.88 ± 5.54	72.48 ± 6.89	61.35 ± 5.88	88.54 ± 8.56	96.36 ± 9.47*	91.36 ± 9.35**	79.36 ± 8.05*
80	68.29 ± 6.84	88.59 ± 8.72	68.53 ± 6.74	95.74 ± 9.47*	99.75 ± 9.86*	98.55 ± 9.64*	98.96 ± 9.74**
100	80.35 ± 8.34	93.26 ± 9.17	77.34 ± 7.62	99.36 ± 9.85*	99.92 ± 9.74*	99.96 ± 9.76*	99.63 ± 9.66*

Table 2: results for SOD Inhibition activity of terpenoids

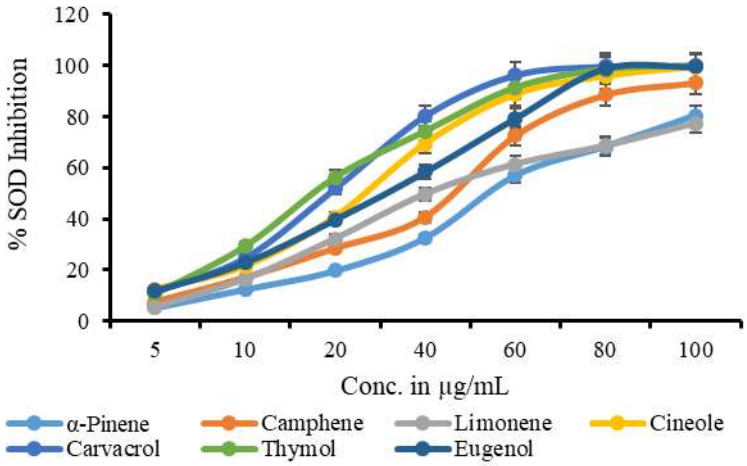


Fig.2: SOD Inhibition activity of terpenoids; Results were represented as mean ± SEM, n=3. ANOVA was used for data analysis; t-test was used to determine the statistical differences between groups. Superscript symbols * and # indicate significant differences observed from either Ascorbic

acid or control group. * Significantly different from control group with $p<0.05$ and ** Significantly different from Ascorbic acid group with $p<0.01$.

3.4 Termites

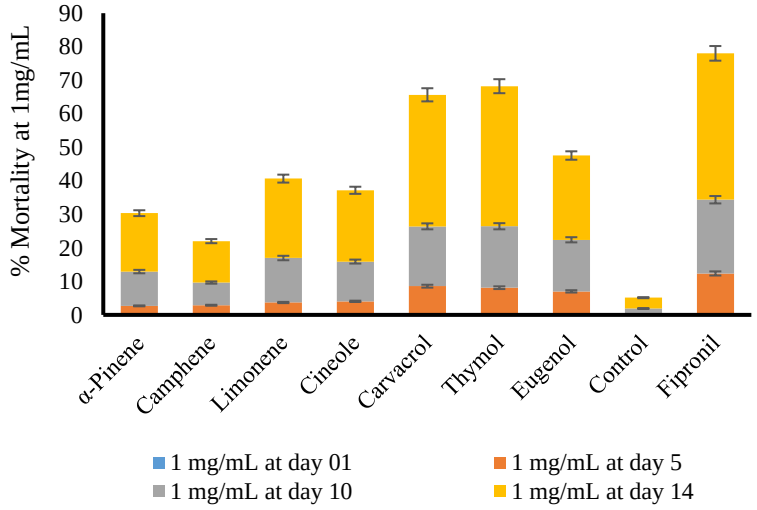
Fig. 3: Indian termite *Odontotermes obesus* grown in humid laboratory conditions

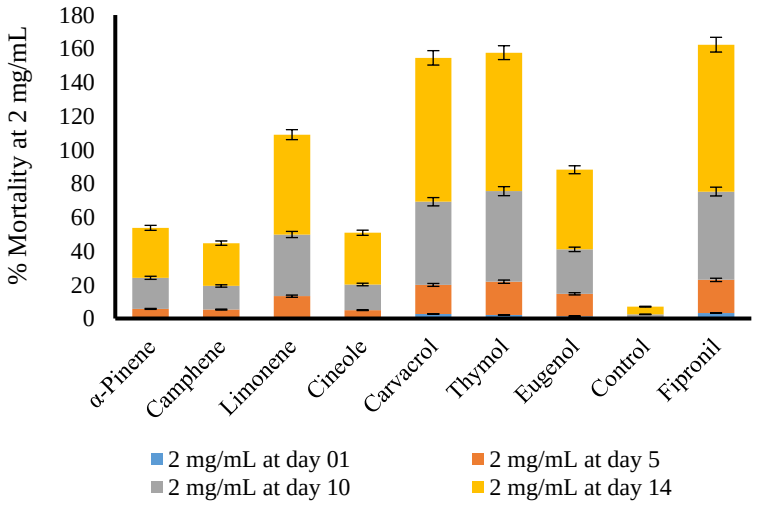
3.5. Anti-termite activity using NO Choice assay

	1 mg/mL at day 01	1 mg/mL at day 5	1 mg/mL at day 10	1 mg/mL at day 14
α-Pinene	0	2.69 ± 0.15	10.23 ± 1.66	17.42 ± 1.23
Camphene	0	2.87 ± 0.19	6.78 ± 0.74	12.35 ± 1.07
Limonene	0	3.69 ± 0.25	13.27 ± 1.17	23.69 ± 1.86
Cineole	0	4.04 ± 0.31	11.86 ± 1.39	21.27 ± 1.96
Carvacrol	0	8.56 ± 0.78	17.85 ± 1.52	39.24 ± 2.35
Thymol	0	8.11 ± 0.96	18.33 ± 1.27	41.78 ± 3.78
Eugenol	0	7.02 ± 0.55	15.38 ± 1.47	25.14 ± 2.55
Control	0	0	1.89 ± 0.28	3.24 ± 0.24
Fipronil	0	12.36 ± 1.08	21.99 ± 1.87	43.67 ± 4.96
	2 mg/mL at day 01	2 mg/mL at day 5	2 mg/mL at day 10	2 mg/mL at day 14
α-Pinene	0	5.69 ± 0.41	18.45 ± 1.89	29.63 ± 2.66
Camphene	0	5.31 ± 0.69	13.99 ± 1.24	25.41 ± 3.05
Limonene	0	13.25 ± 1.12	36.58 ± 2.36	59.22 ± 4.85
Cineole	0	4.96 ± 0.35	15.22 ± 1.08	30.69 ± 2.48
Carvacrol	2.69 ± 0.14	17.24 ± 1.66	49.33 ± 5.23	85.36 ± 7.21
Thymol	2.17 ± 0.16	19.68 ± 1.85	53.68 ± 6.35	82.16 ± 6.25
Eugenol	1.39 ± 0.11	13.24 ± 1.29	26.39 ± 2.78	47.21 ± 3.25

Control	0	0	2.47 ± 0.36	4.56 ± 0.39
Fipronil	3.23 ± 0.47	19.68 ± 1.88	52.38 ± 4.69	87.14 ± 8.52
	3 mg/mL at day 01	**3 mg/mL at day 5**	**3 mg/mL at day 10**	**3 mg/mL at day 14**
α-Pinene	0	12.37 ± 0.96	28.56 ± 2.45	45.87 ± 3.26
Camphene	0	15.22 ± 1.35	32.69 ± 3.06	57.69 ± 3.99
Limonene	5.26 ± 0.42	19.63 ± 1.75	55.38 ± 4.25	85.37 ± 7.42
Cineole	2.69 ± 0.29	13.58 ± 1.22	32.15 ± 3.56	58.61 ± 6.59
Carvacrol	9.33 ± 0.85	42.38 ± 3.69	85.39 ± 7.56	100 ± 5.69
Thymol	10.88 ± 1.24	46.39 ± 4.58	81.37 ± 6.58	100 ± 7.41
Eugenol	5.69 ± 0.47	32.78 ± 2.86	69.23 ± 5.22	91.37 ± 8.23
Control	0	0	4.69 ± 3.21	5.87 ± 4.23
Fipronil	12.36 ± 1.16	46.98 ± 5.27	81.37 ± 7.56	100 ± 8.69

Table 3. Anti-termiticidal activity at different concentrations of terpenoids vs different days.





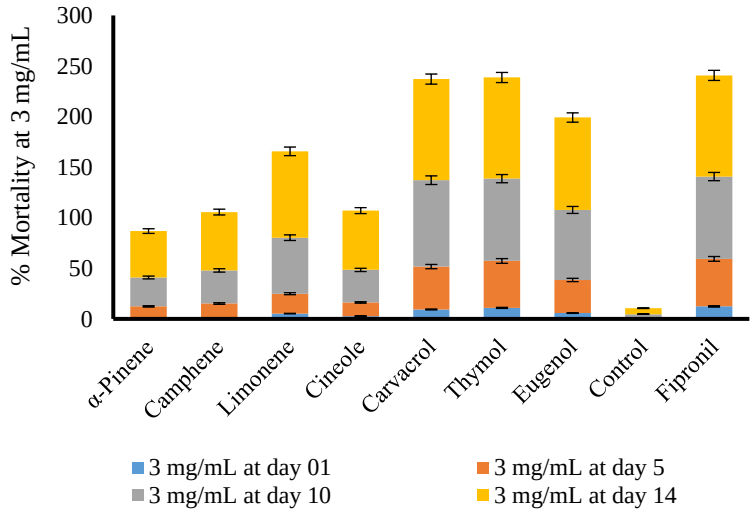


Fig.4: % Mortality of Indian termite in presence of various doses of terpenoids such as 1, 2, and 3 mg/mL of selected compounds; Results were represented as mean ± SEM, n=3. ANOVA was used for data analysis; t-test was used to determine the statistical differences between groups. * Significantly different from control group with $p<0.05$ and ** Significantly different from Ascorbic acid group with $p<0.01$.

% Mass degradation of Cellulose paper Till Day 14			
	1 mg/mL	2 mg/mL	3 mg/mL
α-Pinene	96.32 ± 8.25	79.01 ± 6.33	61.33 ± 4.88
Camphene	93.28 ± 6.39	85.33 ± 6.35	69.35 ± 5.21
Limonene	87.55 ± 8.25	75.34 ± 7.12	51.27 ± 4.21
Cineole	82.39 ± 7.12	71.34 ± 6.58	57.44 ± 4.09
Carvacrol	62.35 ± 6.32	41.72 ± 3.25	23.69 ± 1.68
Thymol	65.98 ± 6.02	38.56 ± 2.89	21.07 ± 1.55
Eugenol	82.35 ± 7.45	70.05 ± 6.59	41.03 ± 3.29
Control	100 ± 9.65	100 ± 9.54	100 ± 8.52
Fipronil	57.66 ± 4.21	38.52 ± 2.87	18.52 ± 1.08

Table 4: % Mass degradation of cellulose paper till 14 day of experimental period

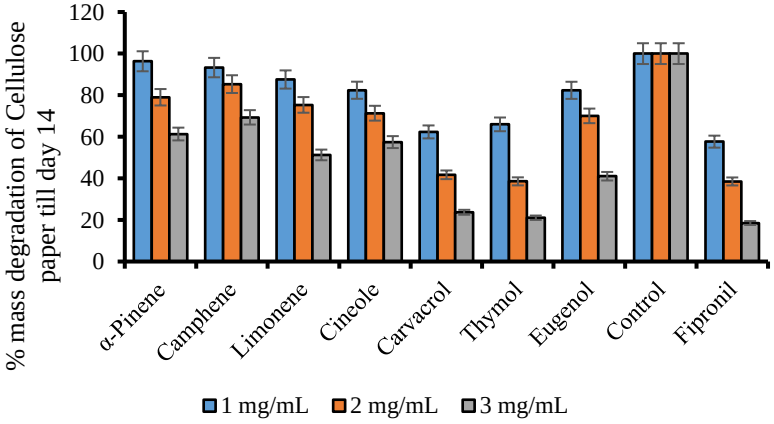
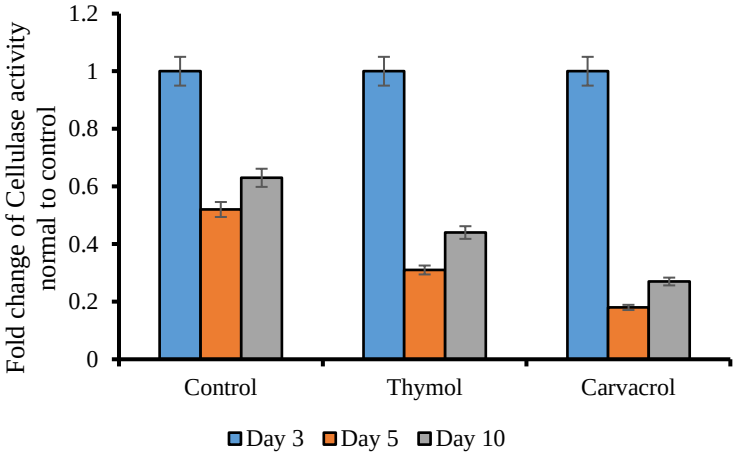


Figure 5: % Mass degradation of Cellulose Paper till Day 14 in presence of various terpenoids

3.6. Enzymatic analysis:

Cellulase activity			
	Day 3	**Day 5**	**Day 10**
Control	1	1	1
Thymol	0.52 ± 0.08	0.31 ± 0.03	0.18 ± 0.02
Carvacrol	0.63 ± 0.05	0.44 ± 0.03	0.27 ± 0.04
β-Glucosidase activity			
Control	1	1	1
Thymol	0.69 ± 0.07	0.47 ± 0.03	0.38 ± 0.05
Carvacrol	0.82 ± 0.06	0.65 ± 0.05	0.51 ± 0.06

Table. Fold change of various enzymes such as Cellulase (a), Beta Glucosidase (b) isolated from Indian termite, *Odontotermes Obesus* when exposed to Thymol, Carvacrol at day 3, 5, and 10.



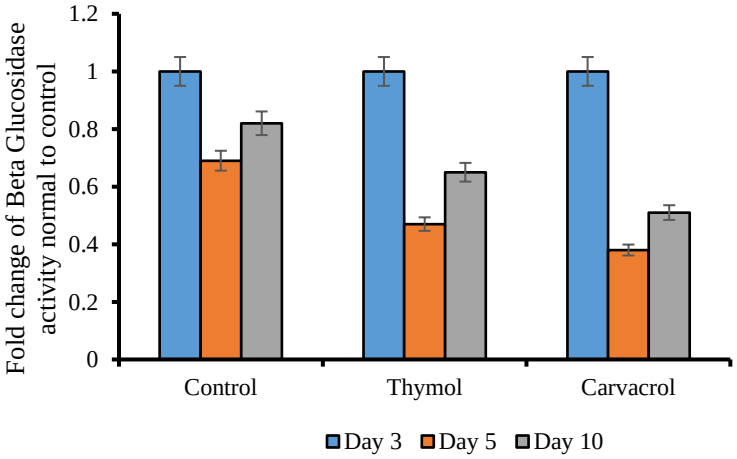
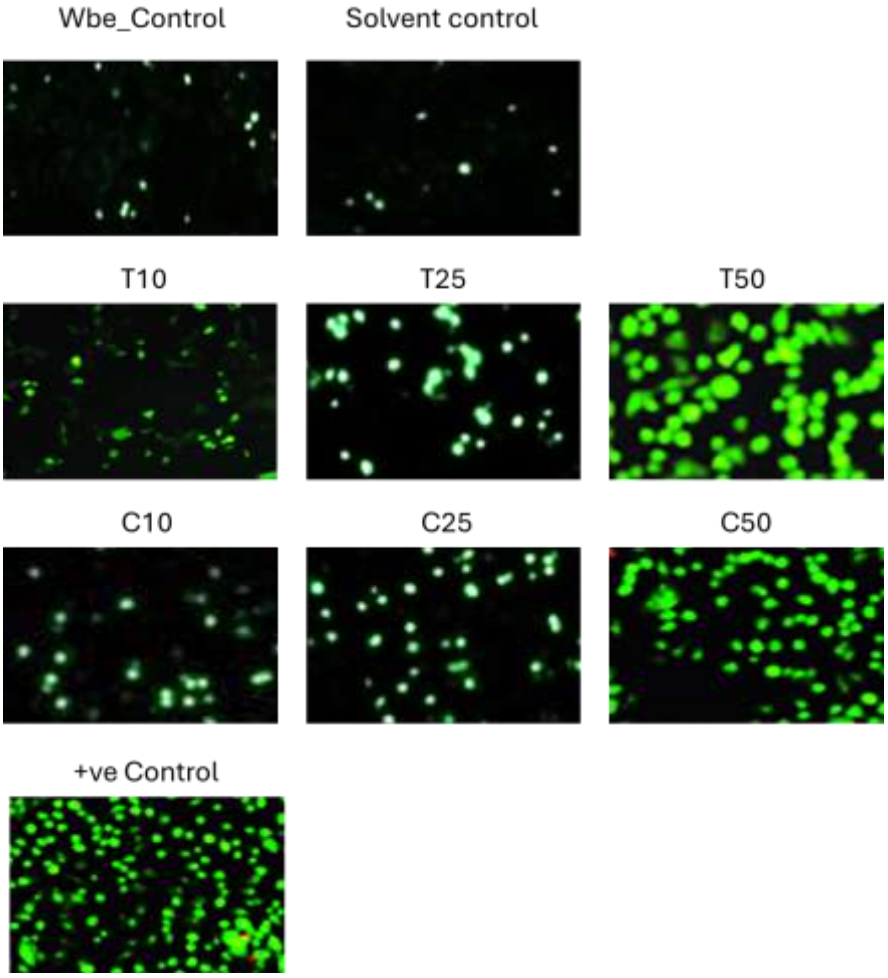


Figure 6: Fold change of various enzymes such as Cellulase (a), Beta Glucosidase (b) isolated from Indian termite, *Odontotermes Obesus* when exposed to Thymol, Carvacrol at day 3, 5, and 10.

3.7. Results of ROS Activation studies

3.7.1. H_2DCFDA assay



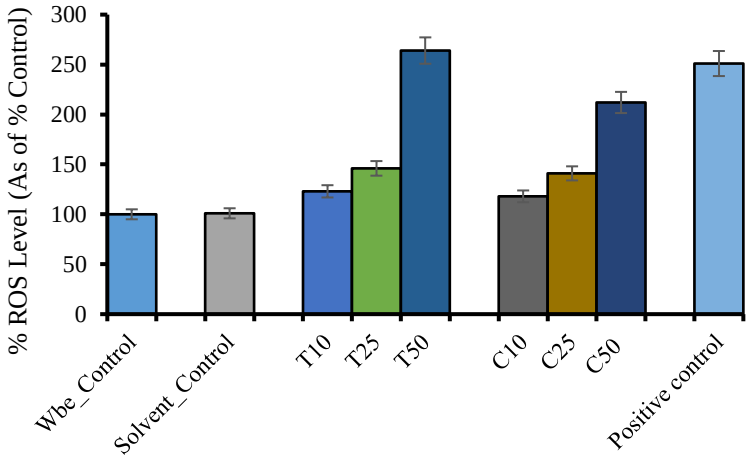


Figure 7: This figure shows the results of the H_2DCFDA assay for nine groups. (a) Fluorescent images indicating ROS determination and (b) quantitative representation of ROS levels; Termites were divided into nine groups of 20 termites each and assigned to normal control (G1), solvent control (G2), thymol treatments (T10: 10 mg/L, G3; T25: 25 mg/L, G4; T50: 50 mg/L, G5), carvacrol treatments (C10: 10 mg/L, G6; C25: 25 mg/L, G7; C50: 50 mg/L, G8), and a positive control (fipronil, G9). The H_2DCFDA assay was performed to measure the relative levels of reactive oxygen species (ROS) in the termites. Higher fluorescence intensity indicates higher ROS levels.

3.7.2. NADPH Oxidase assay

	NADPH Oxidase Activity (nmol/min/mg protein)
Wbe_Control	0.53 ± 0.06
Solvent_Control	0.52 ± 0.04
T10	0.62 ± 0.08
T25	0.83 ± 0.07
T50	1.31 ± 0.09
C10	0.57 ± 0.06
C25	0.78 ± 0.07
C50	1.06 ± 0.09
Positive control	1.42 ± 0.08

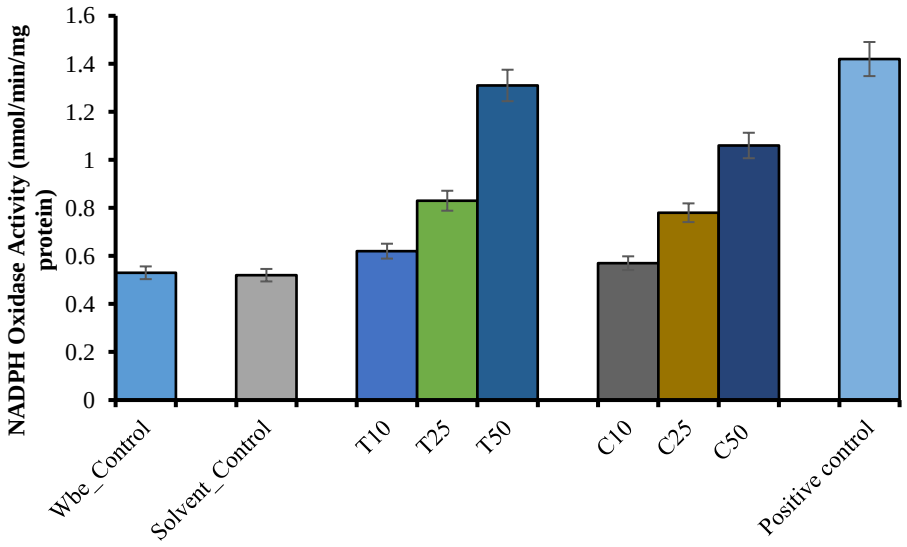


Figure 8: NADPH oxidase activity of Thymol and carvacrol.

In-silico analysis indicates that thymol and carvacrol exert strong binding affinity to the active sites of cellulase and β-glucosidase enzymes in *O. obesus*, the subsequent experimental assays ascertain the inhibitory activity of both enzymes in a concentration-dependent manner. Notably, thymol and carvacrol significantly enhance ROS production in termite cells, suggesting NADPH-mediated oxidative stress may contribute to the inhibition mechanism. The current study thus supports the potent termiticidal activity of thymol and carvacrol against *O. obesus*. Their blockage of the vital digestive enzyme's active sites in cellulose metabolism substantially disrupt termite feeding and metabolism, leading to death. Additionally, the pathological ROS induction mechanism illustrates a two-pronged attack involving direct enzyme inhibition and physiological disruption. Thus, as natural products, thymol and carvacrol are a safe, sustainable alternative to synthetic pesticides with minimal environmental impact.

4. CONCLUSION

we have successfully demonstrated the strong termiticidal effects of thymol and carvacrol against the termite *O. obesus*. The in-silico screening has revealed their remarkable potential to disrupt cellulose metabolism by targeting several digestive enzymes. Furthermore, our determination of the mode of action has confirmed that ROS demonstrates its action through NADPH, which represents a novel target for insecticidal activity. These results provide evidence for thymol and carvacrol as promising alternatives for termite control, which will have a lower ecological footprint compared to conventional pesticides. However, further investigations are required to evaluate their performance in field settings and their side effects on non-target species.

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